OPIC
OFFICE DE LA PROPRIÉTÉ INTELLECTUELLE DU CANADA

CIPO
CANADIAN INTELLECTUAL PROPERTY OFFICE

Ottawa Hull K1A 0C9

(21) (A1) 2,198,474
(86) 1995/08/25
(43) 1996/03/07

(51) Int.Cl.[6] A61K 48/00; A61K 47/42; A61K 31/70

(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Genetic Therapy of Diseases of the Central Nervous System with a Cell-Specific Active Substance Which is Dependent on the Cell Cycle

(72) Sedlacek, Hans-Harald - Germany (Federal Republic of) ;
Müller, Rolf - Germany (Federal Republic of) ;

(71) HOECHST AKTIENGESELLSCHAFT - Germany (Federal Republic of) ;

(30) (GB) 9417366.3 1994/08/26
(GB) 9506466.3 1995/03/29

(57) 17 Claims

Abstract of the disclosure

A DNA sequence is described for the gene therapy of diseases of the central nervous system.

The essential elements of this DNA sequence are the activator sequence, the promoter module and the gene for the active substance.

The activator sequence is activated, in a cell-specific manner, in activated endothelial cells or glial cells. This activation is regulated by the promoter module in a cell cycle-specific manner. The active substance is a nerve growth factor, an enzyme of dopanine metabolism and/or a protective factor for nerve cells. The described DNA sequence is inserted into a viral or non-viral vector which is supplemented by a ligand having affinity for the target cell.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVLEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An active compound for the prophylaxis or therapy of diseases of the central nervous system, which contains a DNA construct which is composed of an activator sequence, a cell cycle-regulated promoter module and a DNA sequence for a neurospecific factor.

2. The active compound as claimed in claim 1), wherein the promoter module possesses the elements CDE-CHR-Inr and contains positions ≤ -20 to ≥ +30 of the cdc25C promoter region (nucleotide sequence (GCTGGCGGAAGGTTTGAATGGTCAACGCCTGCGGCTGTTGATATTCTTG), where CDE constitutes the cell cycle dependent element (nucleotide sequence TGGCGG), CHR constitutes the cell cycle gene homology region (nucleotide sequence GTTTGAA) and Inr constitutes the initiation site (position +1) and also the adjacent sequences which are important for initiation, and also functionally active variants and mutants of the promoter module are likewise included.

3. The active compound as claimed in claim 1), which contains an activator sequence which is regulated by transcription factors which are formed in endothelial cells or in glial cells.

4. The active compound as claimed in claim 2), which contains, as activator sequence,
   - the CMV promoter, the CMV enhancer or the SV40 promoter, or
   - the promoter sequence for the brain-specific, endothelial glucose-1 transporter, for endoglin, for VEGF receptor 1 and 2, the receptor tyrosine kinase til-1 or til-2, the B61 receptor, the B61 ligand for endothelin, in particular endothelin B or endothelin 1, for endothelin receptors, for

mannose 6-phosphate receptor, IL-1α or IL-1β, IL-1 receptor, VCAM-1 or von Willebrand factor, or

- oligomerized binding sites for transcription factors which are preferentially or selectively active in endothelial cells, such as GATA-2 with its binding site 5'-TTATCT-3', or
- promoter sequences for Schwann cell-specific periaxin, for glutamine synthetase, glia-specific protein, the glial cell protein S100b, interleukin-6, 5-hydroxytryptamine receptors, TNFα, IL-10 or insulin-like growth factor receptors, or
- the promoter sequence of VEGF, the enhancer sequence of VEGF or the v-Src DNA sequence or the c-Src DNA sequence, which sequences regulate the VEGF gene.

5. The active compound as claimed in claims 1-3), wherein the DNA sequence for the neurospecific factor encodes

- a neuronal growth factor, in particular FGF, NGF, BDNF, NT-3, NT-4 or CNTF, or
- TGFβ, a soluble TNF receptor, IL-10, a soluble IL-1 receptor or a soluble IL-6 receptor or the IL-1 receptor antagonist or a fusion protein formed from a cytokine (e.g. TGFβ, IL-10 or IL-1 receptor antagonist), or a soluble cytokine receptor (e.g. TNF receptor, IL-6 receptor or IL-1 receptor), and the Fc moiety of human immunoglobulin, or
- a tyrosine hydroxylase or dopa decarboxylase.

6. The active compound as claimed in claim 1-5), which contains the DNA sequences of two identical or two different neurospecific factors, with the two DNA sequences being connected to each other through a DNA sequence for the internal ribosome entry site.

7. The active compound as claimed in claims 1-6), which is inserted into a vector.

8. The active compound as claimed in claim 7), wherein the vector is a virus.

9. The active compound as claimed in claim 8), wherein the virus is a retrovirus, adenovirus, adeno-associated virus, herpes simplex virus or vaccinia virus.

10. The active compound as claimed in claim 1-6), which is inserted into a plasmid.

11. The active compound as claimed in claim 7-10), which is prepared in a colloidal dispersion system.

12. The active compound as claimed in claim 11), wherein the colloidal dispersion system are liposomes.

13. The active compound as claimed in claim 12, wherein the colloidal dispersion system are polylysine ligands.

14. The active compound as claimed in claim 7-13), which is supplemented by a ligand which binds to membrane structures of endothelial cells or glial cells.

15. The active compound as claimed in claim 14), wherein the ligand

- is a polyclonal or monoclonal antibody, or an antibody fragment thereof, which binds, by its variable domains, to membrane structures of endothelial cells or glial cells, or
- is a substance which carries mannose terminally, a cytokine or growth factor or a fragment, or a constituent sequence thereof, which binds to receptors on endothelial cells or glial cells.

16. The active compound as claimed in claim 15), wherein the membrane structures

- constitutes a receptor for a cytokine or a growth factor, such as IL-1, FGF, PDGF, VEGF (in particular FIT-1 and KDR), TGFβ, insulin or insulin-like growth factor (ILGF), or
- constitutes an adhesion molecule, such as SLeX, LFA-1, MAC-1, LECAM-1 or VLA-4
- or constitutes the mannose 6-phosphate receptor.

17. The active compound as claimed in claims 1-16) in a pharmaceutical preparation for intravenous or intra-arterial injection, for local administration at the site of a trauma or for injection into the cavities of the central nervous system.

Gene-therapeutic treatment of diseases of the central nervous system (CNS) with a cell-specific active compound which is dependent on the cell cycle

Technical field

A DNA sequence is described for the gene therapy of diseases of the central nervous system.

The essential elements of this DNA sequence are the activator sequence, the promoter module and the gene for the active substance.

The activator sequence is activated, in a cell-specific manner, in activated endothelial cells or glial cells. This activation is regulated by the promoter module in a cell cycle-specific manner. The active substance is a nerve growth factor, an enzyme of dopanine metabolism and/or a protective factor for nerve cells. The described DNA sequence is inserted into a viral or non-viral vector which is supplemented by a ligand having affinity for the target cell.

1. The central nervous system and growth factors

After the conclusion of ontogenesis, the nerve cells constitute cells which are fully differentiated and no longer capable of division. In general, they are characterized by the nerve cell body and nerve cell processes, with a distinction being made between afferent (dentrites) and efferent (neurites) processes. The efferent neurite, only one of which is generally formed per nerve cell, makes contact with its target orgen (nerve cells or other types of somatic cell) by way of synapses.

Maintenance of the anatomical structure and function of nerve cells is effected in the presence of neuronal growth factors.

In a general sense, neuronal growth factors are to be understood as being neurotrophic factors (Reviews in Massague, Cell 49, 437 (1987), Pusztai et al., J. Pathol. 169, 191 (1993), Ibanez et al., PNAS 89, 3060 (1992), Sonoda et al., BBRC 185, 103 (1992)). These factors include neuronal growth factors in Table 1.

In a narrower sense, the nerve growth factor (NGF) family should be included in these factors.

NGFs act by way of binding to NGF receptors, which are formed, in particular, on the sensory nerve fibers. NGF is taken up intracellularly and transported to the nerve cell body in a retrograde manner (Johnson et al., J. Neurosci. 7, 923 (1987)). In the nerve cell body, the NGF probably brings about an increase in cyclic adenosine monophosphate (cAMP), with subsequently elevated efflux of Ca^{++} (Schubert et al., Nature 273, 718 (1978), and, in addition, release of diacylglycerol and activation of protein kinase C, by way of inositol lipid metabolism, and intracellular release of CA^{++} by way of inositol triphosphate liberation (Abdel-Latif, Pharmacol. Rev. 38, 227 (1986)).

The phosphorylation of specific, in particular signal-transducing, proteins which results from this leads to changes in their function. This results in an increased formation of proteins which are involved in the growth of neurites. These proteins include chartin proteins (Black et al., J. Cell Biol. 103, 545 (1986)) tau proteins and tubulins (Drubin et al., J. Cell Biol. 101, 1799 (1985)). Thus, the synthesis of α-tubulins and β-tubulins, neuro-filament proteins (NF-L, NF-M and NF-H) and peripherin (Portier et al., Devi Neuroscience 6, 215 (1983)), Parysek et al., J Neurosci. 7, 78. (1987)) is elevated. At the same time, the concentration of enzymes which are important in the nervous system, such as choline acetyl-transferase, acetylcholinesterase and neurone-specific enolase (Vinores et al., J. Neurochemistry 37, 597,

1981), Rydel et al., J. Neurosci. 7, 3639 (1987)) increases.

In addition, the concentrations of neurotransmitters, such as neurotensin (Tischler et al., Reg. Pept. 3, 415 (1982)) and neuropeptide Y (Allen et al., Neurosci. Lett. 46, 291 (1984)), and neurotransmitter receptors, such as acetylcholine receptors (Mitsuka et al., Brain Res. 314, 255 (1984)) and encephalin receptors (Inoue et al., J. Biol. Chem. 257, 9238 (1982)) are increased. At the same time, the concentration of synapsin 1 is increased (Romano et al., J. Neurosci. 7, 1300 (1987)).

In the final analysis, NGF maintains the functional state of nerve cells. At the same time, NGF initiates and promotes the growth of neurites. The constant presence of NGF is necessary for this neuritogenic and synaptogenic activity (Smith, Science 242, 708 (1988), Mitchison et al., Neuron 1, 761 (1988)).

This has been reported, in particular, for ciliary neurotrophic factor (CNTF) (Lin et al., Drugs of the Future 19, 557 (1994)).

The neurotrophic activity of neuronal growth factors has been substantiated experimentally, in particular in association with damage to nerve cells, for example in association with the surgical severence of neurites. If CNTF is administered locally to the proximal stump of the transected nerves, the proportion of nerve cells which die following the surgical intervention is markedly reduced (Sendtner et al., Nature 345, 440 (1990)). At the same time, the concentration of, for example, the neuropeptide substance P is markedly elevated in the spinal ganglia following CNTF administration. Rats whose sciatic nerve has been damaged exhibit accelerated restoration of the motor activity following subcutaneous administration of CNTF (Lin et al., Drugs of the Future 19, 557 (1994)).

However, systemic administration of neurotrophic factors is only effective if the motor neurones, which are present in the spinal cord and which are protected by the blood-brain barrier, possess axons which are still functional outside these barriers and by way of which the neurotrophic factors can be taken up (Apfel et al., Brain Res. 605 1 (1993)).

In the case of nerve cell damage up to the other side, or on the other side, of the blood-brain barrier, it is necessary to administer neurotrophic factors intracranially. In this way, retrograde generation of the proximal thalamic neurones following severence of the thalamic axons can, for example, be prevented experimentally (Clatterbuch et al., PNAS 90, 2222 (1993)). However, a prerequisite for an optimal regeneration process is the constant presence of the neutrophic factors at the site of the damaged nerve cell. While it is possible to effect a local administration at the time of the surgical damage or damage relief, this is difficult, or almost impossible to do once the surgical intervention has ended. Diffuse damage to the CNS, for example due to blunt trauma or toxins, affords only very limited opportunity for effecting a targeted administration.

Glial cells can be stimulated to produce TNFα as a result of traumatic, immunological and toxic influences. This TNFα is, at that time, toxic for nerve cells and glial cells, (Owens et al., Immunol. Today 15, 566 (1994)).

In order to achieve a presence of active compounds in the CNS which is as long-term as possible, attempts are made to inject intracranially cells (fibroblasts, endothelial cells and myoblasts) which have been transduced in vitro to express neurotrophic active compounds. The aim is to use the neurotrophic active compounds to improve the regeneration and function of nerve cells which have been damaged traumatically or degeneratively, for example in Parkinson's disease or in dementia. Especially in the

case of Parkinson's disease, attempts are made to inject cells, which either have been transduced in vitro, to secrete neurospecific enzymes such as tyrosine hydroxylase and dopa decarboxylase (Kopin, Ann. Rev. Pharmacol. Toxicol. 32, 467 (1993), Fisher et al., Physiol. Rev. 11, 582 (1993), Jiao et al., Nature 362, 450 (1993)), or else human fetal, dopaminergic, nigral neurones are injected (Löwenstein, Bio/Technology 12, 1075 (1994)).

However, cells of this nature are only available in limited quantities. On the other hand, the use of fetal cells raises important ethical questions.

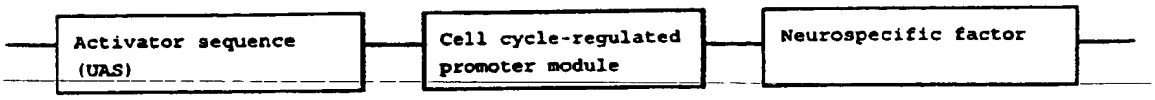
As an alternative, the possibility is being examined of injecting vectors directly into the brain in order to transduce brain cells to express the desired active compounds (During et al., Science 266, 1399 (1994)). However since these vectors do not exhibit any cell specificity, there is the substantial risk of nerve cells being damaged by infection or transfection with the vector.

2. Description of the invention

The present invention now relates to an active compound which can be administered both locally and systemically to patients as a pharmaceutical and by means of which neurospecific factors are produced over a relatively long period at the site of the nerve cell damage. Neurospecific factors of this nature can be neurotrophic factors which protect nerve cells from further damage and bring about regeneration of nerve cells. However, neurospecific factors can also be enzymes, such as tyrosine hydroxylase and dopa decarboxylase, which are responsible for synthesizing dopamine from tyrosine. In addition, neurospecific factors can be substances which inhibit or neutralize TNFα.

The central component of this active compound is a DNA

construct which is composed of the following elements:



(In the entire text of this application, DNA is used as a common term for both a complementary (cDNA) sequence and a genomic DNA sequence).

2.1. Choice of the activator sequence

An activator sequence (UAS = upstream activator sequence) is to be understood as being a nucleotide sequence (promoter sequence or enhancer sequence) with which transcription factors interact which are formed or are active in endothelial cells or glial cells.

The CMV enhancer or CMV promoter (EP-B1-0 173 177), or the SV40 promoter, or any other promoter sequence or enhancer sequence which is known to the skilled person, can be used as the activator sequence.

However, within the meaning of this invention, the preferred activator sequences include gene-regulatory sequences or elements from genes which encode proteins which are formed, in particular, in endothelial cells or glial cells.

a) Activator sequences which are activated in endothelial cells

Some of these proteins have been described by Burrows et al. (Pharmac. Therp. 64, 155 (1994) and Plate et al. (Brain Pathol. 4, 207 (1994)). In particular, these endothelial cell-specific proteins include, for example:

- Brain-specific, endothelial glucose-1 transporter

Endothelial cells of the brain express this transporter very strongly for the purpose of achieving the transendothelial transport of D glucose into the brain (Gerhart et al., J. Neurosci. Res. 22, 464 (1989)). The promoter sequence has been described by Murakami et al. (J. Biol. Chem. 267, 9300 (1992)).

- Endoglin

Endoglin appears to be a TGFβ receptor which is not signal-transmitting (Gangos et al., J. Biol. Chem. 265, 8361 (1990), Moren et al., BBRC 189, 356 (1992), Cheifetz, J. Biol. Chem. 267, 19027 (1992)). While it is present in small quantities on normal endothelium, it is expressed more strongly on proliferating endothelium (Westphal et al., J. Invest. Derm. 100, 27 (1993), Burrows et al., Pharmac. Ther. 64, 155 (1994)). The promoter sequences have been described by (Bellon et al., Eur. J. Immunol. 23, 2340 (1993), Ge et al., Gene 138, 201 (1994)).

- VEGF receptors

Two receptors are distinguished (Plate et al., Int. J. Cancer 59, 520 (1994)).

* VEGF receptor 1 (flt-1) (de Vries et al., Science 255, 989 (1992)) contains an fms-like tyrosine kinase in the cytoplasmic moiety, and

* VEGF receptor 2 (flk-1, KDR) (Terman et al., BBRC 187, 1579 (1992)) contains a tyrosine kinase in the cytoplasmic moiety.

Both receptors are found almost exclusively on endothelial cells (Senger et al., Cancer Metast. Rev. 12, 303 (1993)).

- Other endothelium-specific receptor tyrosine kinases

* til-1 or til-2 (Partanen et al., Mol. Cell Biol. 12, 1698 (1992), Schnürch and Risau, Developm. 119, 957 (1993), Dumont et al., Oncogene 7, 1471 (1992))

* B61 receptor
(Eck receptor) (Bartley et al., Nature 368, 558 (1994), Pandey et al., Science 268, 567 (1995), Van der Geer et al., Ann. Rev. Cell Biol. 10, 251 (1994))

- B61
The B61 protein is the ligand for the B61 receptor. (Holzman et al., J. Am. Soc. Nephrol. 4, 466 (1993), Bartley et al., Nature 368, 558 (1994))

- endothelin, in particular

* Endothelin B (Oreilly et al., J. Cardiovasc. Pharm. 22, 18 (1993), Benatti et al., J. Clin. Invest. 91, 1149 (1993), O'Reilly et al., BBRC 193, 834 (1993)). The promoter sequence has been described by Yanasigawa et al., Nature 332, 411 (1988) and Benatti et al., J. Clin. Invest. 91, 1149 (1993).

* endothelin-1 (Yanasigawa et al., Nature 332, 411 (1988)). The promoter sequence has been described by Wilson et al., Mol. Cel.. Biol. 10, 4854 (1990).

* endothelin receptors, in particular the endothelin B receptor (Webb et al., Mol. Pharmacol. 47, 730 (1995), Haendler et al., J. Cardiovasc. Pharm. 20, 1 (1992)).

- Mannose 6-phosphate receptors
(Perales et al., Eur. J. Biochem. 226, 225 (1994), Dahms et al., Cell 50, 181 (1987)).

The promoter sequences have been described by Ludwig et al., (Gene 142, 311 (1994)), Oshima et al., (J. Biol. Chem. 263, 2553 (1988)) and Pohlmann et al. (PNAS USA 85, 5575 (1987)).

- von Willebrand factor
The promoter sequence has been described by Jahroudi and Lynch (Mol. Cell. Biol. 14, 999 (1994), Ferreira et al., Biochem. J. 293, 641 (1993) and Aird et al., PNAS USA 92, 4567 (1995)).

- IL-1α and IL-1β
IL-1 is produced by activated endothelial cells (Warner et al., J. Immunol. 139, 1911 (1987).
The promoter sequences have been described by Hangen et al., Mol. Carcinog. 2, 68 (1986), Turner et al., J. Immunol. 143, 3556 (1989), Fenton et al., J. Immunol. 138, 3972 (1987), Bensi et al., Cell Growth Diff. 1, 491 (199'), Mori et a'., Blood 84, 1688 (1994), Hiscott et al., Mol. Cell. Biol. 13, 6231 (1993).

- IL-1 receptor
The promoter sequence has been described by Ye et al., PNAS USA 90, 2295 (1993).

- Vascular cell adhesion molecule (VCAM-1)
The expression of VCAM-1 in endothelial cells is activated by lipopolysaccharides, TNF-α (Neish et al., Mol. Cell. Biol. 15, 2558 (1995)), IL-4 (Iademarco et al., J. Clin. Invest. 95, 264 (1995)), IL-1 (Marni et al., J. Clin. Invest. 92, 1866 (1993)).
The promoter sequence of VCAM-1 has been described by Neish et al., Mol. Cell. Biol. 15, 2558 (1995), Ahmad et al., J. Biol. Chem. 270, 8976 (1995), Neish et al., J. Exp. Med. 176, 1583 (1992), Iademarco et al., J. Biol. Chem. 267, 16323 (1992) and Cybulsky et al., PNAS USA 88, 7859 (1991).

- Synthetic activator sequences

Synthetic activator sequences, which are composed of oligomerized binding sites for transcription factors which are preferentially or selectively active in endothelial cells, can also be used as an alternative to natural endothelium-specific promoters. An example of these synthetic activator sequences is transcription factor GATA-2, whose binding site in the endothelin-1 is gene...TTATCT... (Lee et al., Biol. Chem. 266, 16188 (1991); Dorfmann et al., J. Biol. Chem. 267, 1279 (1992) and Wilson et al., Mol. Cell. Biol. 10, 4854 (1990)).

b) Activator sequences, activated in glial cells

A preferred activator sequence is furthermore to be understood as being a nucleotide sequence (promoter sequence or enhancer sequence) which interact with transcription factors which are formed to a particularly great extent, or are active, in glial cells.

These activator sequences include, in particular, gene-regulatory sequences or elements from genes which, for example, encode the following proteins which can be detected in glial cells:

* The Schwann cell-specific protein periaxin (Gillespie et al., Neuron 12, 497 (1994))
  The promoter sequences have been described by Gillespie et al., (Neuron 12, 497 (1994)).

* Glutamine synthetase
  (Akimoto et al., Brain Res. 72, 9 (1993), Fressinaud et al., J. Cell Physiol. 149, 459 (1991)).
  The promoter sequences have been described by Chakrabarti et al. (Gene 153, 163 (1995)) and Bhandarie et al., (J. Biol. Chem. 266, 7784 (1991)).

* The glial cell-specific protein
(Glial fibrillary acidic protein = GFAP)
(Akimoto et al., Brain Res. 72, 9 (1993))
The promoter sequences have been described by Kumanishi et al. (Acta Neuropath. 83, 569 (1992)), Besuard et al. (J. Biol. Chem. 266, 18877 (1991)), Reeves et al. (PNAS USA 86, 5178 (1989)), Brenner et al. (Brain Res. 7, 277 (1990)) and Masood et al. (J. Neurochem. 61, 160 (1993)).

* The S100b glial cell protein
(Shen et al., Mol. Brain Res. 21, 62 (1994))
The promoter sequence has been described by Zimmer et al., (Brain Res. Bulletin 37, 417 (1995)).

* IL-6 (CNTF)
(Sparacio et al., J. Neuroimmunol. 39, 231 (1992))
The promoter sequences have been described by Chernajoosky et al. (J. Cell. Biochem. Suppl. 0/13, 73 (1989)), Ray et al. (Mol. Cell Biol. 10, 5736 (1990)), Droogmans et al. (DNA Sequence 3, 115 (1992)), Mori et al. (Blood 84, 2904 (1994)), Liberman et al. (Mol. Cell. Biol. 10, 2327 (1990)) and Ishiki et al. (Mol. Cell. Biol. 10, 2757 (1990)).

* 5-HT receptors
(Whitaker-Azmitia et al., Synapse 14, 201 (1993))
The promoter sequences have been described by Elliott et al. (Neurochem. Int. 25, 537 (1994)), Veldman et al. (Mol. Pharmac. 42, 439 (1992)), Adham et al. (PNAS USA 90, 408 (1993)), Mochizuki et al. (BBRC 185, 517 (1992)) and Jin et al. (J. Biol. Chem. 267, 5735 (1992)).

* TNFα
(Perez et al., Cell 63, 251 (1990), Merrill et al., J. Immunol. 151, 2132 (1993)).
The promoter sequences have been described by Takashiba et al. (Gene 131, 307 (1993)) and van der Ahe et al. (Nucl. Acids Res. 21, 5636 (1993)).

* IL-10
(Owens et al., Immunol. Today 15, 566 (1994)).
The promoter sequences have been described by Kim et al. (J. Immunol. 148, 3618 (1992)), Kube et al. (Cytokine 7, 1 (1995) and Platzer et al. (DNA-Sequence 4, 399 (1994)).

* Insulin-like growth factor receptor I and II
The promoter sequences have been described by Morgan et al. (Nature 329, 301 (1987)), Cooke et al. (BBRC 177, 1113 (1991)), Kim et al. (Mol. Endocrin. 5, 1964 (1991)), van Dijk et al. (Mol. Cell. Endocrin. 81, 81 (1991)), Raizis et al. (Biochem. J. 289, 133 (1993)) and Yu et al. (Nature 371, 714 (1994)).

- VEGF
VEGF is formed in vascularized tissue, particularly under hypoxic conditions (Berse et al., Mol. Biol. Cell 3, 211 (1992), Finkenzeller et al., BBRC 208, 432 (1995), Tischer et al., BBRC 165, 1198 (1989), Leung et al., Science 246, 1306 (1989), Ferrara et al., Endoc. Rev. 13, 18 (1992)). The gene-regulatory sequence for the VEGF gene is

  * The promoter sequence of VEGF (5' flanking region)
  Michenko et al., Cell Mol. Biol. Res. 40, 35 (1994), Tischer et al., J. Biol. Chem. 266, 11947 (1991) or

* the enhancer sequence of the VEGF gene (3' flanking region) (Michenko et al., Cell Mol. Biol. Res. 40, 35 (1994)) or

* the c-Src gene (Mukhopadhyay et al., Nature 375, 577 (1995), Bonham et al., Oncogene 8, 1973 (1993), Parker et al., Mol. Cell. Biol. 5, 831 (1985), Anderson et al., Mol. Cell. Biol. 5, 1122 (1985)) or

* the v-Src gene (Mukhodpadhyay et al., Nature 375, 577 (1995), Anderson et al., Mol. Cell. Biol. 5, 1122 (1985), Gibbs et al., J. Virol. 53, 19 (1985))

2.2. Choice of the promoter module

A cell cycle-regulated promotor module is, for example, to be understood as being the nucleotide sequence -CDE-CHR-Inr-. The essential function of the promotor module is to inhibit the function of the activator sequence in the G0/G1 phase of the cell cycle and to ensure cell cycle-specific expression in the S/G2 phase, and consequently in proliferating cells.

The promoter module CDE-CHR-Inr was discovered in the context of a detailed investigation of the G2-specific expression of the human cdc25C promoter. The starting point was finding a regulatory element ("cell cycle dependent element"; CDE) which is responsible for switching off the promoter in the G1 phase of the cell cycle (Lucibello et al., EMBO J. 14, 132 (1995)). Using genomic dimethyl sulfate (DMS) footprinting and functional analyses (Figs. 1 and 2), it was demonstrated that the CDE binds a repressor ("CDE-binding factor"; CDF) in a G1-specific manner and thereby leads to inhibition of transcription in non-proliferating (G0) cells. The CDE,

which is located within the region of the basal promoter, is dependent, in its repressing function, on an "upstream activating sequence" (UAS).

This led to the conclusion that the CDE-binding factor inhibits the transcription-activating effect of 5'-bound activator proteins in a cell cycle-dependent manner, i.e. in non-proliferating cells and in the G1 phase of the cell cycle (Fig. 3).

This conclusion was confirmed by a further experiment: fusion of the viral, non-cell cycle-regulated early SV40 enhancer to a cdc25 minimum promoter (composed of CDE and the 3' situated start sites) led to clear cell cycle regulation of the chimeric promoter (Fig. 4). Subsequent examination of the cdc25C enhancer has demonstrated that the transcription factors which are regulated by the CDF in a cell cycle-dependent manner are NF-Y (CBF) (Dorn et al., Cell 50, 863 (1987), van Hujisduijnen et al., EMBO J. 9, 3119 (1990), Coustry et al., J. Biol. Chem. 270, 468 (1995)), Sp1 (Kadonaga et al., TIBS 11, 10 (1986)) and a transcription factor (CIF) which is possibly novel and which binds to CBS7. Another interesting finding of this study was the observation that NF-Y only activates transcription efficiently within the cdc25C enhancer in cooperation with at least one further NF-Y complex or with CIF. Both NF-Y and Sp1 belong to the glutamine-rich activator class, which provides important indications with regard to the mechanism of the repression (e.g. interaction or interference with particular basal transcription factors or TAFs).

A comparison of the promoter sequences of cdc25C, cyclin A and cdc2 demonstrated homologies in several regions (Fig. 5). This is not only the CDE, but also the adjacent Yc boxes, which are conserved in all 3 promoters (the divergences which exist are not functionally relevant). As expected, all these regions exhibited protein binding in vivo, with this protein binding taking place

in a cell cycle-dependent manner in the case of the CDE. In addition, it was demonstrated that all 3 promoters are deregulated by a mutation of the CDE (Table 2). A remarkable similarity was also evident when comparing the cdc25C, cyclin A and cdc2 sequences in the region immediately 3' of the CDE ("cell cycle genes homology region"; CHR) (Fig. 5). While this region is functionally as important as the CDE (Table 2), it is not visible in the in-vivo DMS footprinting experiments.

A possible explanation for this is an interaction of the factor with the minor groove of the DNA. Results of "electrophoretic mobility shift assay" (EMSA) experiments indicate that CDE and CHR together bind a protein complex, the CDF. These observations suggest the conclusion that CDF-mediated repression of glutamine-rich activators is a frequently occurring mechanism of cell cycle-regulated transcription.

However, it is apparently not only the CDE-CHR region which is of importance for regulating the cdc25C promoter but also one of the initiation sites (position +1) within the nucleotide sequence of the basal promoter (positions $\leq$ -20 to $\geq$ +30, see Fig. 1). Mutations in this region, which encompasses the in-vitro binding site for the transcription factor YY-1 (Seto et al., Nature 354, 241 (1991), Usheva and Shenk Cell 76, 1115 (1994)), lead to complete deregulation. In view of the proximity of the CDE-CHR to the basal promoter, interaction of the CDF with the basal transcription complex is consequently very probable.

2.3. Choice of the neurospecific factor

a) Neuronal growth factors

Within the meaning of the invention, a neurospecific factor is to be understood as being a DNA sequence which encodes a neuronal growth factor. By way of

example, these neuronal growth factors include, in particular:

- FGF
  (Johnson et al., Adv. Cancer Rec. 60, 1 (1993), Jay et al., Science 233, 541 (1986), Abrahahm et al., EMBO J. 5, 2523 (1986), Science 233, 545 (1986), Mergia et al., BBRC 138, 644 (1986), Schweigerer, Nature 325, 257 (1987), PNAS USA 84, 842 (1987))

- Nerve growth factor (NGF)
  (Haktzopoulous et al., Neuron 13, 187 (1994), Takeda et al., Neuroscience 55, 23 (1993), Cartwright et al., Brain Res. 15, 67 (1992))

- Brain-derived neurotrophic factor (BDNF)
  (Zhang et al., J. Neurobiol. 25, 1517 (1994), Maisonpierre et al., Genomics 10, 558 (1991), DNA Sequence 3, 49 (1992), Timmusk et al., Neuron 10, 475 (1993)).

- Neurotrophin-3 (NT-3)
  (Hallboeoek et al., Eur. J. Neurosci. 5, 1 (1993), Rodriguez-Tebar et al., Philiosoph. Transact. Roy. Soc. Biol. Sci. 331, 255 (1991), Leingärtner et al., Eur. J. Neurosci. 6, 1149 (1994))

- Neurotrophin-4 (NT-4)
  (Ibanez et al., PNAS 89, 3060 (1992), Ny et al., PNAS 89, 3060 (1992))

- Ciliary neurotrophic factor (CNTF)
  (Ishiki et al., New Biologist 3, 63 (1991), Ray et al., Mol. Cell Biol. 9, 5537 (1989), Leung et al., Neuron 8/6, 1045 (1992), Bootha et al., Gene 146, 303 (1994)).

b) Enzymes

In addition, a neurospecific factor is to be understood as being a cDNA sequence which encodes:

- tyrosine hydroxylase
  (Goc et al., Mol Cell Neurosci. 3, 383 (1992), Boularand et al., J. Biol. Chemistry 270, 3748 (1995))

or

- dopa decarboxylase
  (Maras et al., Eur. J. Biochem. 201, 385 (1991), Nayatsu, Neurosci. Res. 12, 315 (1991), Ichinose et al., Biochem. 31, 11546 (1992), Levanthai et al., Mol. Brain Res. 17, 227 (1993), Sumiichinose et al., J. Neurochem. 64, 514 (1995)).

c) Cytokines and their inhibitors

A neurospecific factor is furthermore to be understood as being a DNA sequence which encodes proteins which inhibit or neutralize the neurotoxic effect of TNFα. These proteins include, for example:

- TGFβ
  (Massague, Ann. Rev. Cell. Biol. 6, 597 (1990), Kondiah et al., J. Biol. Chem. 265, 1089 (1990), Garnier et al., J. Mol. Biol. 120, 97 (1978)). TGFβ inhibits TNFα-mediated cytotoxicity (Merrill et al., J. Immunol. 151, 2132 (1993), Quin et al., Annals of Surgery 220, 508 (1994))

- Soluble TNF receptors
  (Nophar et al., EMBO J. 9, 3269 (1990), Himmler et al., DNA Cell Biol. 9, 705 (1990), Aggarwal et al., Nature 318, 665 (1985), Gray et al., PNAS 87, 7380 (1990), Tartaglia et al., Immunol. Today 13, 151 (1992), Loetcher et al., Cell 61, 351

(1990), Schall et al., Cell 61, 361 (1990), Smith et al., Science 248, 1019 (1990), Goodwin et al., Mol. Cell. Biol. 11, 3020 (1991)).
TNF receptors neutralize TNFα. Review: Olsson et al., Eur. Cytokine Netw. 4, 169 (1993).

- IL-10
(Moore et al., Science 248, 1230 (1990), Vieira et al., PNAS USA 88, 1172 (1991), Kim et al., J. Immunol. 148, 3618 (1992)).
IL-10 inhibits the formation of IFN gamma, TNFα, IL-2 and IL-4 (Schlaak et al., Scand. J. Immunol. 39, 209 (1994), Vieira et al., PNAS USA 88, 1172 (1991), Benjamin et al., Leuk. Lymph. 12, 205 (1994))

- soluble IL-1 receptors
  * IL-1 receptor I
  (Sims et al., PNAS USA 86, 8946 (1989), Dower et al., J. Exp. Med. 162, 501 (1985), Chizzonite et al., PNAS 86, 8029 (1989)

  * IL-1 receptor II
  (McMahan et al., EMBO J. 10, 2821 (1991), Sims et al., Science 241, 585 (1988)).
  Soluble IL-1 receptors neutralize the activity of IL-1 (Colotta et al., Immunol. Today 15, 562 (1994), Sims et al., Clin. Immunol. Immunopath. 72, 9 1994))

- IL-1 receptor antagonist
(Eisenberg et al., Nature 343, 341 (1990), Carter et al., Nature 344 (633 (1990))

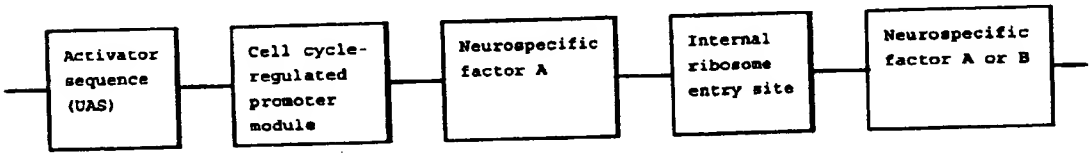
- soluble IL-6 receptors
(Mackiewicz et al., Cytokine 7, 142 (1995))

However, within the meaning of the invention, DNA sequences of fusion proteins formed between the listed

cytokines and growth factors, or the extracellular moiety of the receptors, on the one hand, and the Fc moiety of human immunoglobulin, on the other hand, can also be used as the active substance. DNA sequences of this nature, and their preparation have been described in EP 0 464 633 A1.

2.4. Combination of several neurospecific factors

The invention furthermore relates to an active compound in which a combination of the DNA sequences of identical neurospecific factors (A,A) or different neurospecific factors (A,B) is present. The cDNA of an "internal ribosome entry site" (IRES) is preferably interpolated, as a regulatory element, for the purpose of expressing two DNA sequences.



IRESs of this nature have, for example, been described by Montford and Smith (TIG 11, 179 (1995), Kaufman et al., Nucl. Acids Res. 19, 4485 (1991), Morgan et al., Nucl. Acids Res. 20, 1293 (1992, Dirks et al., Gene 128, 247 (1993), Pelletier and Sonenberg, Nature 334, 320 (1988) and Sugitomo et al., BioTechn. 12, 694 (1994).

Thus, the cDNA of the IRES sequence of poliovirus (position _< 140 to _> 630 of the 5' UTR (Pelletier and Sonenberg, Nature 334, 320 (1988)) can be used for linking the DNA of antiinflammatory substance A (at the 3' end) and the DNA of antiinflammatory substance B (at the 5' terminus).

Depending on the combination, an active compound of this nature exhibits either an additive (A+A, A+B1) or a synergistic effect within the meaning of the invention.

2.5. Construction of the vector

The novel DNA construct is made into a vector in a manner familiar to the skilled person. This vector can be of viral or non-viral origin. For example, the novel DNA construct is inserted into a viral vector (in this regard, see D. Jolly, Cancer Gene Therapy 1, 51 (1994)), or else completed to form a plasmid. Viral vectors or plasmids can be complexed with collodial dispersions. These dispersions include, for example, liposomes (Farhood et al., Annals of the New York Academy of Sciences 716, 23 (1994)) or else polylysine/ligand conjugates (Curiel et al., Annals of the New York Academy of Sciences 716, 36 (1994)).

2.6. Choice of the ligands

Viral and non-viral vectors can be supplemented with a ligand. Substances which bind to the surface of endothelial cells are preferred as ligand, for example in polylysine/ligand conjugates. These substances include antibodies or antibody fragments which are directed against membrane structures of endothelial cells, as described, for example, by Burrows et al. (Pharmac. Ther. 64, 155 (1994)) or in EP 0 408 859 A2. In particular, these substances include antibodies against the VEGF receptors.

The murine monoclonal antibodies should preferably be employed in humanized form. The humanization is effected in the manner described by Winter et al. (Nature 349, 293 (1991) and Hoogenboom et al. (Rev. Tr. Transfus. Hemobiol. 36, 19 (1993). Antibody fragments are prepared in accordance with the state of the art, for example in the manner described by Winter et al., Nature 349, 293 (1991), Hoogenboom et al., Rev. Tr. Transfus. Hemobiol. 36, 19 (1993), Givol, Mol. Immunol. 28, 1379 (1991) or Huston et al., Int. Rev. Immunol. 10, 195 (1993).

These substances additionally include all active compounds which bind to membrane structures or membrane receptors on endothelial cells. For example, the active compounds include growth factors, or their fragments or constituent sequences thereof, which bind to receptors which are expressed by endothelial cells, such as, for example, PDGF, bFGF, VEGF and TGFβ (Pusztai et al., J. Pathol. 169, 191 (1993)). In addition, these substances include substances which carry mannose terminally and bind to the mannose 6-phosphate receptor of endothelial cells (Perales et al., Eur. J. Biochem. 226, 225 (1994)).

In addition, these substances include adhesion molecules which bind to activated and/or proliferating endothelial cells. Adhesion molecules of this nature, such as SLeX, LFA-1, MAC-1, LECAM-1 or VLA-4, have already been described (reviews in Augustin-Voss et al., J. Cell Biol. 119, 483 (1992), Pauli et al., Cancer Metast. Rev. 9, 175 (1990), Honn et al., Cancer Metast. Rev. 11, 353 (1992)).

In addition, substances which bind to the surface of glial cells are to be regarded as ligands.

These substances include antibodies or antibody fragments which are directed against membrane structures of glial cells, as reported, for example, by Mirsky et al., (Cell and Tissue Res. 240, 723 (1985) by Coakham et al., (Prog. Exp. Tumor Rex. 29, 57 (1985)) and by McKeever et al. (Neurobiol. 6, 119

(1991)). These membrane structures additionally include neural adhesion molecules such as N-CAM, in particular its polypeptide chain C (Nybroe et al., J. Cell Biol. 101, 2310 (1985)).

These substances additionally include all active compounds which bind to membrane structures or membrane receptors on glial cells. For example, these substances include substances which carry mannose terminally and

which bind to the mannose 6-phosphate receptor (Perales et al., Eur. J. Biochem. 226, 225 (1994), insulin and insulin-like growth factor (Merrill et al., J. Clin. Endocrin. Metab. 71, 199 (1990)), PDGF (Ek et al., Nature 295, 419 (1982)) and those fragments of these growth factors which bind to the relevant membrane receptors.

2.7. Preparation of the active compound

Preparation of the novel active compound is described in more detail with the aid of the following example:

a) Construction of the chimeric promoter endothelin 1CDE-CHR-Inr

The human endothelin-1 promoter (position ≤ -170 to ≥ -10) , or a variant which is truncated by the TATA box (position ≤ -170 to ≥ -40), is linked, at its 3' end, to the 5' terminus of the CDE-CHR-Inr module (position ≤ -20 to ≥ +121) of the human cdc25C gene (Fig. 6). The linking is effected using enzymes which are known to the skilled person and which are commercially available.

b) Construction of a plasmid which contains the central component of the active compound

The chimeric endothelin-1 repressor module transcription unit which has been prepared in this way is linked at its 3' ends to the 5' terminus of a DNA which contains the complete coding region of the IL-1 receptor antagonist 152 amino acids in length (DNA position ≤ 25 to ≥ 557; Eisenberg et al., Nature 343, 341 (1990)). This DNA also contains the signal sequence (25 N-terminal amino acids) which is necessary for secretion. Transcription control units and IL-1 receptor antagonist DNA are cloned into pUC19/19 or Bluescript-derived plasmid vectors which can be used, either directly (Yovandich et al., Hum. Gene Ther. 6 603 (1995)) or in colloidal dispersion systems, for an in-vivo transfer.

Alternatively, the transcription control units and IL-1 receptor antagonist DNA which have been joined together can be transferred into viral vectors or other non-viral vectors which are familiar to the skilled person.

2.8. Activity of the active compound

Following local administration, for example at the site of the nerve damage or intracranial or subarachnoid administration, or systemic, preferably intravenous or intraarterial, administration, an active compound according to the present invention enables, by means of the tissue-specific enhancer and the basal promoter, endothelial cells, which are mainly, if not exclusively, only proliferating cells, or proliferating glial cells to secrete neurospecific factors. Endothelial cell proliferations or glial cell proliferations of this nature are to be expected in the region and as a reaction to tissue damage which has concomitantly also caused the nerve damage. The novel active compound consequently ensures a high concentration of the neurospecific factor at the site of the nerve damage.

Since the active compound promises a high degree of safety, both on account of its cell specificity and its cell cycle specificity, it can also be used for the prophylaxis or therapy of nerve damage in high doses and, if necessary, repeatedly at intervals of days or weeks.

Legends to Figs. 1-6:

Fig. 1:
Nucleotide sequence of the cdc25C promoter region showing the protein binding sites (genomic DMS footprinting; • (filled circles): complete constitutive protection; o (open circle): partial constitutive protection; * (asterisk): cell cycle-regulated, G1-specific protection) which were found *in vivo*. CBS: constitutive binding site; CDE: cell cycle-dependent element. Regions which are

underlaid in gray indicate the Y_c boxes (NF-Y binding sites). Start sites are labelled by filled squares.

Fig. 2:
Derepression of the cdc25C promoter specifically in G_0 by means of mututating the cdc.

Fig. 3:
Diagrammatic representation of the regulation of the cdc25C enhancer by the CDE.

Fig. 4:
G_0/G_1-specific repression of the SV40 enhancer by the CDE.

Fig. 5:
Homologies in the CDE-CHR region and the 5'-situated Yc boxes in the cdc25C, cyclin A and cdc2 promoters

Fig. 6:
Chimeric constructs composed of different moieties of the human endothelin-1 promoter, the 3'-fused promoter module containing the CDE and CHR repressor elements, and also a DNA for the IL-1 receptor antagonist (complete coding region, position ≤ 25 to ≥ 557; Eisenberg et al., Nature 343, 341 (1989)), as effector. Position indications relate to the data of Wilson et al., Mol. Cell. Biol. 10, 4854 (1990) for the endothelin-1 gene or to the system used by Lucibello et al., EMBO J. 14, 132 (1995) for cdc25C.

Table 1: Neuronal growth factors

	Site of formation	Site of action
Epidermal growth factor family		
Schwannoma-derived growth factor (SDGF)	Schwann cells	Astrocytes Schwann cells Fibroblasts
Heparin binding growth factor family		
acidic fibroblast growth factor (aFGF)	ubiquitous	ubiquitous
basic fibroblast growth factor (bFGF)	ubiquitous	ubiquitous
Nerve growth factor family		
nerve growth factor (NGF)	Schwann cells Neurones Melanocytes Cholinergic neurones in the brain	Peripheral neurones
brain derived neurotrophic factor (BDNF)	Neurones Glial cells	Dopaminergic neurones in the brain
Neurotrophin-3, -4 (NT-3, NT-4)	many cell types	Peripheral proprioceptive neurones
Ciliary neurotrophic factor (CNTF)		Peripheral nerve cells

Table 2: Role of CDE and CHR in the cell cycle-regulated transcription of cdc25C, cyclin A and cdc2

Tab. 2

	G_0	Growing	Factor
wt			
cdc25C	0.8	13.1	17.5
cyclin A	0.7	27.1	41.7
cdc2	1.0	41.2	41.2
mCDE(-13)			
cdc25C	7.6	11.6	1.5
cyclin A	13.4	23.9	1.8
cdc2	11.3	33.9	3.0
mCHR(-6/-3)			
cdc25C	14.4	21.0	1.5
cyclin A	15.5	28.3	1.8
cdc2	18.6	38.6	2.1

The results of transient transfections in HIH3T3 cells are presented as RLUs/1000. mCDE: mutated CDE (pos. -13: G → T); mCHR: mutated CHR (pos. -6 to -3).

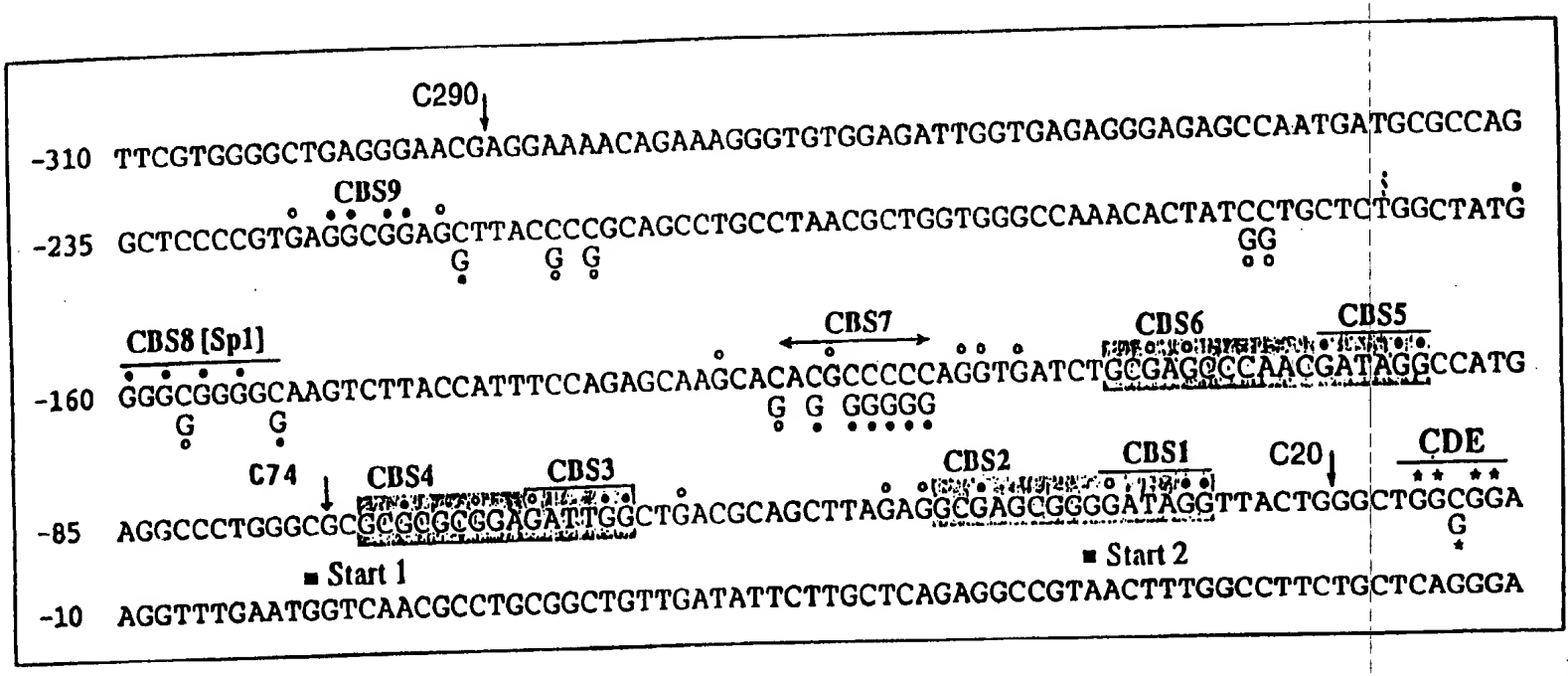

C290
-310 TTCGTGGGGCTGAGGGAACGAGGAAAACAGAAAGGGTGTGGAGATTGGTGAGAGGGAGAGCCAATGATGCGCCAG
CBS9
-235 GCTCCCCGTGAGGCGGAGCTTACCCCGCAGCCTGCCTAACGCTGGTGGGCCAAACACTATCCTGCTCTGGCTATG
CBS8 [Sp1]
CBS7
CBS6
CBS5
-160 GGGCGGGGCAAGTCTTACCATTTCCAGAGCAAGCACACGCCCCCAGGTGATCTGCGAGCCCAACGATAGGCCATG
C74
CBS4
CBS3
CBS2
CBS1
C20
CDE
-85 AGGCCCTGGGCGCGCGCGCGGAGATTGGCTGACGCAGCTTAGAGGCGAGCGGGGATAGGTTACTGGGCTGGCGGA
Start 1
Start 2
-10 AGGTTTGAATGGTCAACGCCTGCGGCTGTTGATATTCTTGCTCAGAGGCCGTAACTTTGGCCTTCTGCTCAGGGA

Fig. 1

2198474

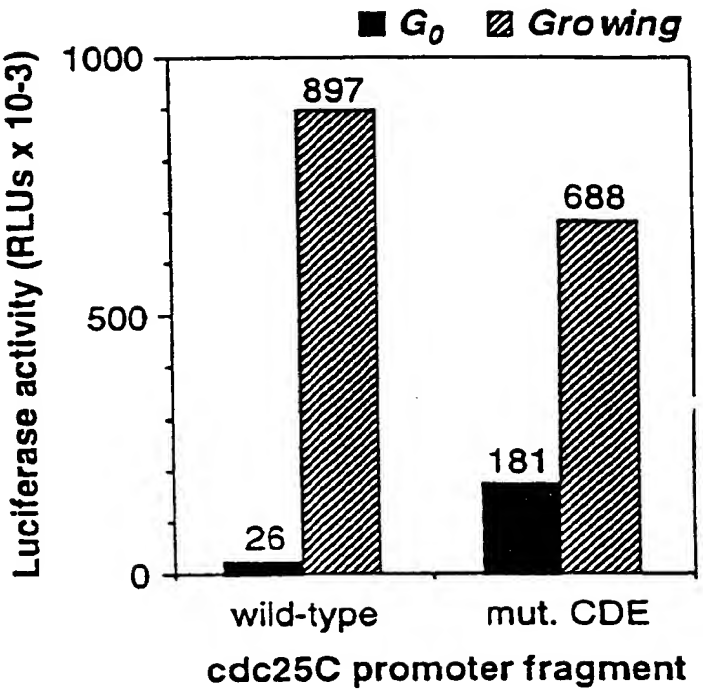


Fig. 2

2198474

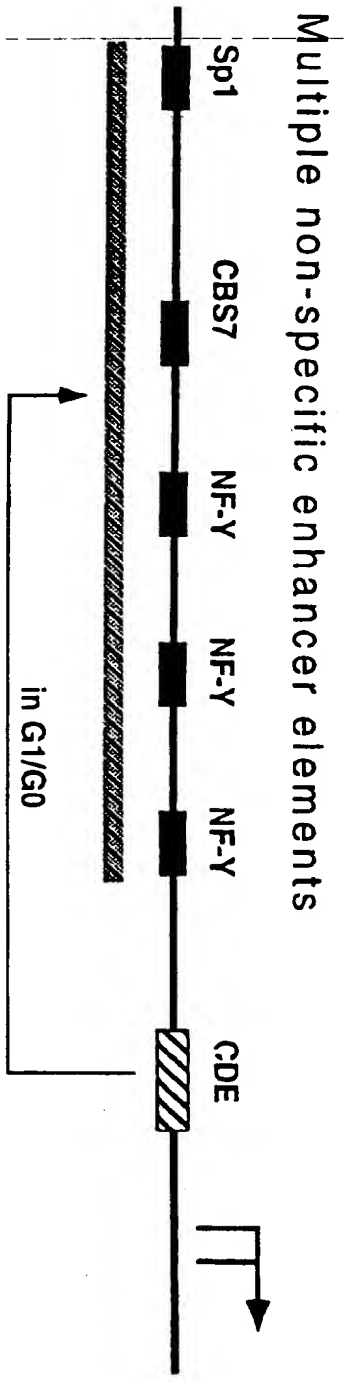


Fig. 3

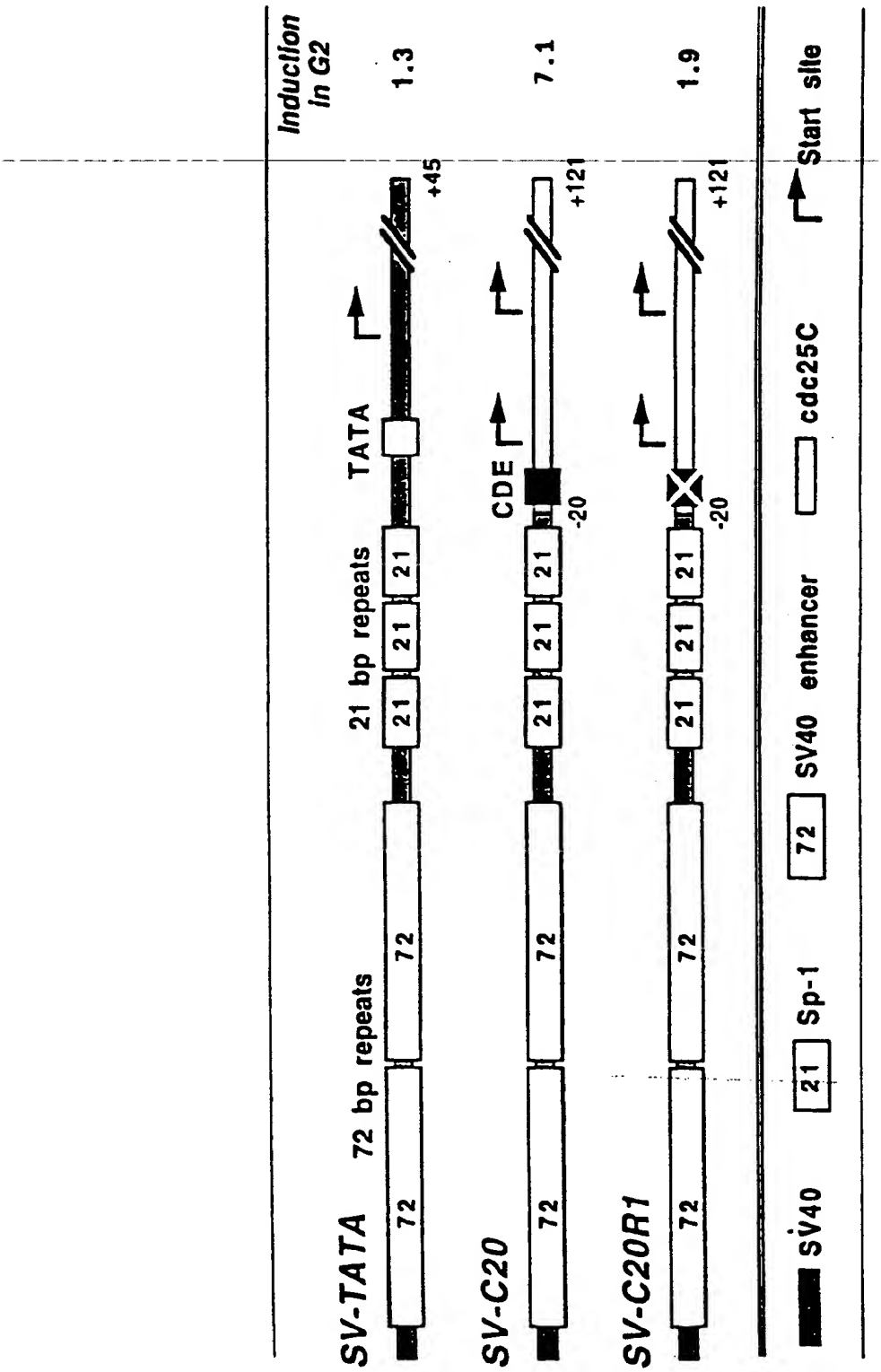

Induction in G2
1.3
7.1
1.9
SV-TATA
72 bp repeats
TATA
+45
21 bp repeats
21
21
21
72
72
SV-C20
CDE
+121
-20
21
21
21
72
72
SV-C20R1
+121
-20
21
21
21
72
72
SV40
21
Sp-1
72
SV40 enhancer
cdc25C
Start site

Fig. 4

cdc25C
cdc2
cyclin A
Yc-Box 2
Yc-Box 1
CTGTCGCCTTGAATGACGTCA
ATF
9 bp
10 bp
6 bp
8 bp
17 bp
9 bp
CDE - CHR
CDE - CHR
CDE - CHR

Fig. 5

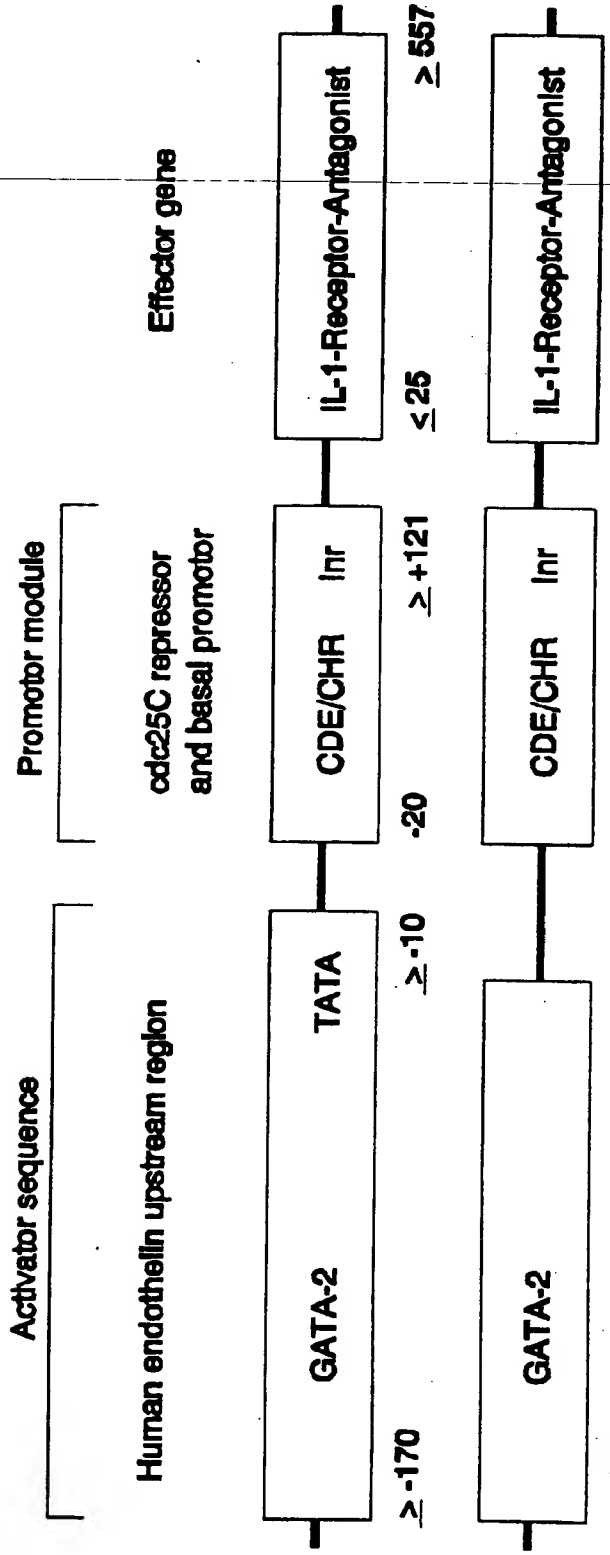

Activator sequence
Human endothelin upstream region
GATA-2
TATA
≥ -170
≥ -10
Promotor module
odc25C repressor and basal promotor
CDE/CHR
Inr
-20
≥ +121
Effector gene
IL-1-Receptor-Antagonist
≤ 25
≥ 557
GATA-2
≥ -170
≥ -40
CDE/CHR
Inr
IL-1-Receptor-Antagonist

Fig. 6